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Programmed Cell Death in Zebrafish Rohon Beard Neurons Is Influenced by TrkC1/NT-3 Signaling[1]

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Rohon Beard (RB) cells are embryonic primary sensory neurons that are removed by programmed cell death during larval development in zebrafish. RB somatosensory functions are taken over by neurons of the dorsal root ganglia (DRG), suggesting that RB cell death may be triggered by the differentiation of these ganglia, as has been proposed to be the case in *Xenopus*. However, here we show that the timing of RB cell death correlates with reduced expression of *trkC1*, the receptor for neurotrophin NT-3, but not with the appearance of DRG, which differentiate only after most RB cells die. *trkC1* is expressed in subpopulations of RB neurons during development, and cell death is initiated only in *trkC1*-negative neurons, suggesting a role for TrkC1 and its ligand, NT-3, in RB cell survival. In support of this, antibodies that deplete NT-3 induce RB cell death while exogenous application of NT-3 reduces death. In addition, we show that RB cell death can be prevented using a caspase inhibitor, zVADfmk, showing that during normal development, RB cells die by a caspase-dependent programmed cell death pathway possibly triggered by reduced signaling via TrkC1.

Key Words: **Rohon Beard neurons; programmed cell death; TrkC1; NT-3.**

INTRODUCTION

In many animals, a primary neural network formed early in development controls embryonic and early larval senses and movements, while a later developing neural network serves the animal throughout the remainder of its life. Primary neurons are lost (Lamborghini, 1987), reutilized (Alley *et al.*, 1998), or retained (Mendelson, 1986; Williams and Shepherd, 1999) depending upon their function. In animals that undergo metamorphosis, including insects and frogs, hormones such as ecdysone and thyroxine, respectively, are thought to be triggers for primary neuronal cell death. However, little is known about the signals that coordinate the switch from primary to secondary neurons in animals that do not undergo metamorphosis.

An example of the switch from primary to secondary neurons is the transition from Rohon Beard (RB) sensory neurons to dorsal root ganglia (DRG). RB cells are the first sensory neurons to develop in the trunk and are removed by cell death during larval development in fish and frogs (Lamborghini, 1987; Bernhardt *et al.*, 1990). The free nerve endings of RB neurons detect light touch stimuli and initiate the first escape reaction in the embryo (Clarke *et al.*, 1984). The sensory functions of these CNS neurons are replaced by neurons of the DRG, which constitute the main somatosensory system in later life. RB neurons are the largest cells in the embryonic spinal cord and form a nearly continuous double row along its dorsal surface. Each cell has axons that project rostrally to the hindbrain and caudally within the spinal cord within a small dorsolateral fiber tract. Eventually the RB axons terminate near the otocyst in the hindbrain and in the caudal end of the spinal cord (Bernhardt *et al.*, 1990; Metcalfe *et al.*, 1990). Each RB cell also has a peripheral axon that runs over or through the segmental myotomes to innervate the skin. RB cells are not segmentally distributed and their peripheral sensory arbors penetrate multiple segments (Bernhardt *et al.*, 1990). DRG neurons have similar axonal projections despite their cell bodies lying in peripheral ganglia rather than in the CNS (Bernhardt *et al.*, 1990). Thus, despite the different locations

[1] This paper is dedicated to the memory of Nigel Holder who died tragically during the course of this project.

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of RB and DRG cell bodies, they have similar central and peripheral targets.

In lampreys and newts, RB cells remain functional throughout adult life despite the development of a secondary sensory system (Nakao and Ishikawa, 1987). However, in most species, RB cells die. In *Xenopus*, RB neurons undergo two waves of cell death (Lamborghini, 1987), the first of which occurs when DRGs differentiate and the second just before metamorphic climax. In this case, competition between RB neurons and DRG neurons may be the cause for the first wave of death while the second wave of death may reflect a surge of thyroxine at metamorphic climax (Lamborghini, 1987).

Neuronal cell number is finely tuned during embryonic and larval development by the process of apoptosis (Oppenheim, 1991). Apoptosis or programmed cell death is a controlled form of cell death involving cascades of degenerative events. Proteolysis during apoptosis is largely due to the activity of a family of cysteine proteases called caspases. Caspases are involved in a cascade: a proapoptotic signal can activate an initiator caspase that subsequently activates effector caspases, resulting in cellular disassembly. Different initiator caspases mediate distinct sets of signals. For example caspase-8 is associated with apoptosis signaled through the Fas receptor and the tumor necrosis factor receptor (TNFR). Activation of procaspase-8 requires association with its cofactor FADD via the death domain (DD) of the receptors (Koseki *et al.*, 1998). Also, within the nervous system, the neurotrophin receptor p75 (p75 NTR) can induce caspase-independent programmed cell death upon ligand withdrawal (Lievremont *et al.*, 1999). The intracellular pathway by which p75 NTR activates caspases is unknown. However, p75 NTR contains a region of sequence homology with DDs which could activate caspases (Chapman, 1995) and TNFR-associated factors have been shown to interact with p75 NTR (Ye *et al.*, 1999), suggesting that signaling via the TNFR pathway may be involved.

Strong candidates for the survival factors required by RB neurons and DRGs are neurotrophins. Neurotrophic factors are critical for the development, survival, and regeneration of the nervous system and their loss leads to the death of subsets of neurons (Davies, 1994). Major actions of neurotrophins are mediated by a family of receptor tyrosine kinases consisting of TrkA, TrkB, and TrkC. Each neurotrophin and Trk receptor can also interact with p75 NTR (Bibel *et al.*, 1999). The neurotrophins are thought to regulate neuronal survival during the period of target innervation through being available in limiting amounts (Oppenheim, 1991). For example, early neuronal dependence on NT-3 has been studied extensively in mouse trigeminal ganglia (Paul and Davies, 1995; Wilkinson *et al.*, 1996). Trigeminal ganglia initially survive independent of neurotrophins, then display a transient dependency on BDNF and NT-3 which is lost as the neurons become NGF dependent. It is after this change in neurotrophin dependence, when the levels of neurotrophins are not high enough to maintain all the neurons, that many of the cells die.

Five Trk receptors have been cloned in zebrafish (Martin *et al.*, 1995) and it has been shown that at 24 h postfertilization (hpf) *trkC1* is expressed in a subset of RB neurons (Martin *et al.*, 1998). Here we show that none of the RB cells that are undergoing apoptosis are *trkC1* positive, suggesting a role for its ligand, NT-3 in the survival of the RB cells. We show that an antibody that blocks NT-3 function can increase the number of dying RB neurons and that exogenous NT-3 is able to decrease the number of RB neurons undergoing programmed cell death. Furthermore the hypothesis that DRG compete with RB neurons for a survival factor (which could be NT-3), causing the death of the RB cells, seems incompatible with the observation that most RB cell death occurs before DRG differentiation.

MATERIALS AND METHODS

Maintenance of Zebrafish

A breeding colony of zebrafish (*Danio rerio*) was maintained at 28.5°C on a 14-h-light/10-h-dark cycle (Westerfield, 1995). All embryos were collected by natural spawning and staged according to Kimmel *et al.* (1995). Embryos were generated from the wild-type line *ucl.*

Administering Caspase Inhibitor zVADfmk to Zebrafish

To ensure penetration of the caspase inhibitor zVADfmk (benzyloxycarbonyl Val-Ala-Asp-(*O*-methyl)fluoromethyl ketone), embryos were incubated in concentrations of zVADfmk of 0 to 1000 μM in fish water for 12 h. A control peptide for zVADfmk, zFAfmk (benzyloxycarbonyl-Phe-Ala-(*O*-methyl)fluoromethyl ketone), was administered to the embryos as above. Both zVADfmk and zFAfmk were obtained from Enzyme Systems Products (Dublin, CA). For the majority of experiments zVADfmk was used at a concentration of 300 μM.

Detection of Apoptotic Cell Death

To determine patterns of programmed cell death, embryos were staged and collected from 50% epiboly to 24 h with intervals of 1 h and from 24 to 48 hpf with intervals of 2 h. Terminal transferase dUTP nick-end labeling (TUNEL) was used to determine cell death using a modification of the protocol suggested by the manufacturer (ApopTag *in Situ* Apoptosis Detection Kit–Peroxidase; Oncor, Inc.; Williams and Holder, 2000).

Whole-Mount in Situ Hybridization

Whole-mount *in situ* hybridization was carried out as described in Thisse *et al.* (1993). *trkC1* cDNA used was a gift from Dr. Stella Martin and used as in Martin *et al.* (1995).

Labeling Rohon Beard and DRG Neurons

Rohon Beard neurons were detected by labeling with HNK-1 antibody (Sigma) at a concentration of 1/1000. Anti-mouse IgM

HRP conjugated was used as secondary antibody. Standard procedures were used for antibody labeling of embryos ranging from 12 somites to 48 hpf (Wilson *et al.*, 1990). For double labeling cells with HNK-1 and TUNEL, embryos first underwent HNK-1 antibody staining followed by TUNEL. For double labeling with HNK-1 and *trkC1*, embryos first underwent HNK-1 antibody labeling followed by *in situ* hybridization. Procedures for labeling were the same as above with the exception that 1 μl/ml RNasin and 500 μM torula RNA were included in the blocking and PBT washes during antibody labeling. For triple labeling with anti-HNK-1, *trkC1* RNA probe, and TUNEL, embryos were first doubled labeled with HNK-1 antibody and RNA probe as above. After overnight fixing in 4% paraformaldehyde, the TUNEL protocol was carried out as above, omitting the ethanol/acetic acid postfixation stage.

DRG were detected by immunostaining with Hu antibody (Molecular Probes) used at a concentration of 1/1000. Anti-mouse IgG HRP conjugated was used as secondary antibody. Standard antibody labeling protocol was used on embryos at the stages of 36, 48, and 60 hpf (Wilson *et al.*, 1990). To increase permeabilization after fixation, embryos were treated with methanol at −20°C for 1 h and proteinase K treated (30 mg/ml) for 10 min at room temperature.

In Vitro Culture of Trunk Explants of Zebrafish Embryos

Embryos were grown to 16 somites in fish water. They were dechorionated with forceps and placed in a petri dish of Ringer's solution (116 mM NaCl, 2.9 mM KCl, 1.8 mM $CaCl_2$, 5 mM Hepes, pH 7.2). The yolk was removed with forceps and the head was cut off. Next, the explant was placed into growth medium (L-15 (Sigma), 0.3 mg/ml glutamine (Imperial Laboratories), 50 U/ml (Imp. Laboratories), 0.05 mg/ml streptomycin (Imp. Laboratories), 0.8 mM $CaCl_2$, 3% fetal calf serum (Gibco)) and oriented with the ventral side down. Most of the medium was then removed using a very fine glass Pasteur pipette and a drop of agarose (1% low-melting-point agarose in growth medium) at 35°C added onto the explant. When set, another drop of agarose was added followed by growth medium. Explants were then incubated at 28°C.

Administering NT-3 and Anti-NT-3 to Explants

Embryos staged at 16 somites were dissected into explants and cultured on agar in growth medium. Each explant was put in a well and exposed to a range of concentrations of either anti-NT-3 (0.5–200 ng/ml) or NT-3 (0.05–50 ng/ml), obtained from PeproTechEC. The explants were incubated for 4 or 6 h at 28.5°C. The explants were removed, washed in Ringer's, and fixed overnight in 4% paraformaldehyde at 4°C.

Photography and Microscopy

Embryos stained after antibody, *in situ* hybridization, or TUNEL reactions were fixed in 4% paraformaldehyde in PBS for 1 h at room temperature, washed in PBT, and then cleared successively in 30, 50, and then 70% glycerol in PBS. Specimens were examined using a compound microscope (Nikon) equipped with DIC optics and photographed using EKTAR 25 film (Kodak).

RESULTS

Generation and Loss of RB Cells Occur between 15 hpf and 3–4 Days of Development

Differentiated RB cells can be identified by their dorsal positions in the spinal cord, large size, axonal trajectories, and labeling by HNK-1. To determine the time course of RB cell loss and generation, we counted the total numbers of RB neurons after labeling with HNK-1 antibody at different stages between 15 and 84 hpf (Figs. 1A–1C and 1F).

HNK-1-labeled RB cells are first detected at the 12-somite stage (15 hpf), when there are 30 ± 5 labeled cells (n = 20 embryos). This number increases, reaching a peak of 190 ± 6.2 (n = 20 embryos) cells by 34 hpf. RB cells then gradually decrease in number at an average rate of about 5–6 cells/h from 34 hpf and later 2–3 cells are lost hourly between 48 and 60 hpf. In the majority of the embryos, all RB cells are lost by 3.5 days (Fig. 1F) but in a minority of cases RB cells were detected as late as 5 days of age.

RB Neurons Die by Caspase-Dependent Apoptosis prior to Differentiation of DRG

To determine if RB cells die by apoptosis, we labeled fragmented DNA of dying cells with TUNEL. At the 20-somite stage (19 hpf) this technique revealed a few large-bodied dying cells along the dorsal spinal cord and by 26 h the dying cells form an easily visible row (Fig. 2A). Dying RB cells still have processes that are labeled with HNK-1 antibody, and double staining with HNK-1 antibody and TUNEL confirmed that the parallel rows of cells along the medial dorsal surface of the spinal cord are RB neurons undergoing programmed cell death (Fig. 2B).

TUNEL labeled only a subpopulation of RB cells. At 26 hpf this subpopulation represents about 20% of the RB neurons, and this proportion changed with time until virtually all RB cells were apoptotic by 3 days (Fig. 1F). These results suggest that most or all RB cells are lost by apoptosis between 19 hpf and 3.5 dpf.

To determine if the RB cells die by caspase-dependent PCD, zebrafish embryos were treated with a caspase inhibitor (zVADfmk). Embryos incubated from bud stage (10 hpf) to 26 hpf with caspase inhibitor zVADfmk (300 μM) exhibited a significant increase in the number of RB neurons and a reduced number of TUNEL-labeled cells (Figs. 2D and 2E), in comparison with untreated and zFAfmk-treated controls (Figs. 2B, 2C, and 2E). For example, at 26 hpf, embryos treated with 300 μM zVADfmk had 140 ± 7 HNK-1-positive cells (n = 20), 5% of which were TUNEL positive, while untreated embryos had 128 ± 6 HNK-1$^+$ cells (n = 20), of which an average of 20% were dying, and zFAfmk-treated controls had 126 ± 6 HNK-1$^+$ cells (n = 20) of which an average of 19% were dying (Fig. 2E). Thus blocking caspase activity significantly decreases RB cell death ($P < 0.05$), showing that RB cells die by a caspase-dependent programmed cell death pathway.

Caspase inhibitor-treated embryos have a decrease in

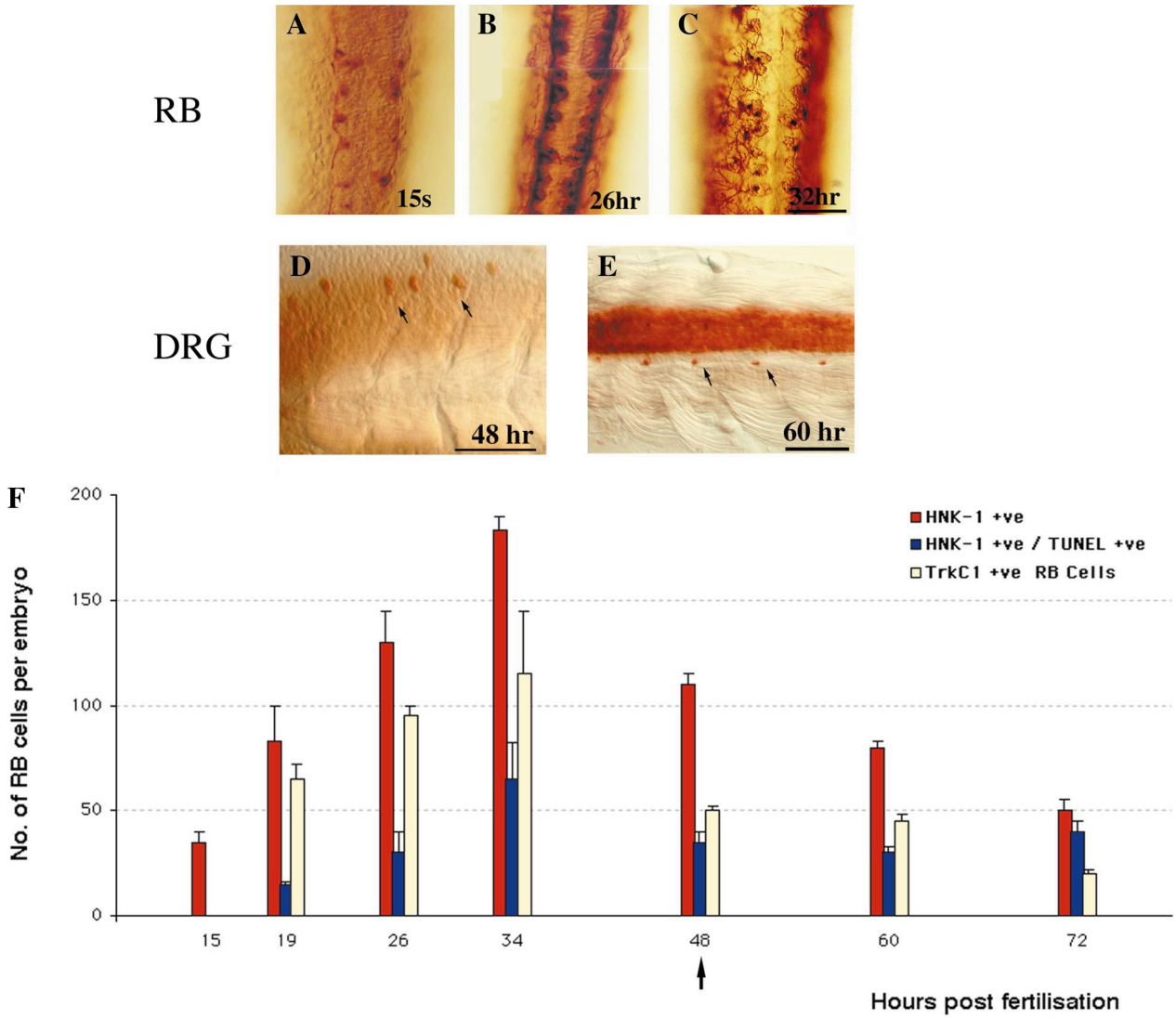


FIG. 1. Rohon Beard cell generation and loss during early development. (A–C) HNK-1-labeled RB cells in spinal cords of zebrafish embryos at 15 s (A), 26 hpf (B), and 32 hpf (C). Dorsal views with anterior up. Scale bar, 50 μm. (D and E) Anti-Hu-labeled DRG at 48 (D) and at 60 hpf (E). At 48 hpf, only a small number of neurons (arrows) are labeled in the most anterior DRG, whereas by 60 hpf, one or two neurons are labeled in each of the DRG segmentally positioned along the trunk (arrows). Lateral views with anterior to the left. (F) Graph showing the total number of HNK-1$^+$ differentiated RB cells (red), the number of apoptotic (TUNEL-labeled) RB cells (blue), and the number of *trkC1*-positive RB cells (yellow) per embryo over time. Values represent means ± SEM. Arrow indicates the stage at which DRG neurons start to differentiate.

number of TUNEL-positive cells at 26 hpf. However, when examined at 48 hpf, caspase inhibitor-treated embryos showed no significant reduction in dying cells compared to untreated and zFAfmk-treated controls (Fig. 2E) and by 3.5 days, most or all RB cells were removed. This recovery of death was observed even in embryos that were repeatedly treated with the caspase inhibitor. These observations suggest either that the cells eventually die by a caspase-independent pathway or that at later developmental stages, zVADfmk is unable to penetrate into the spinal cord.

To examine the temporal relationship between RB cell death and DRG differentiation, we determined the time course of differentiation of DRG. Using an antibody to the Hu protein, an early marker of cells undergoing neurogenesis (Wakamatsu *et al.*, 1997), we first observed neurons within DRG at 48 hpf. At this stage there were one or two neurons in each of the first ganglia to differentiate adjacent to the rostral-most somites (Fig. 1D). By 60 hpf, one or two neurons were detected in each of the DRG segmentally distributed along the full extent of the trunk (Fig. 1E), supporting previous observa-

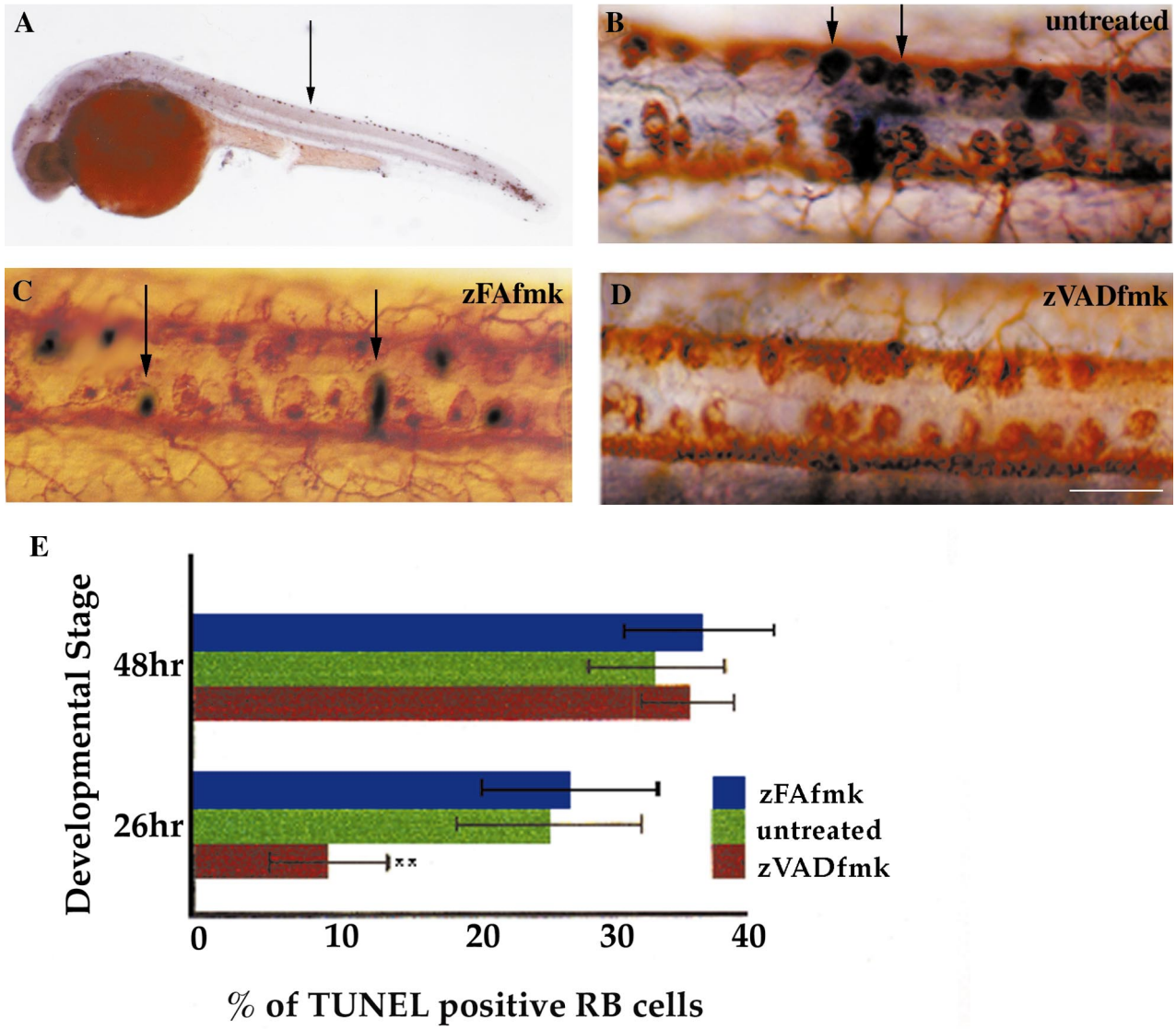
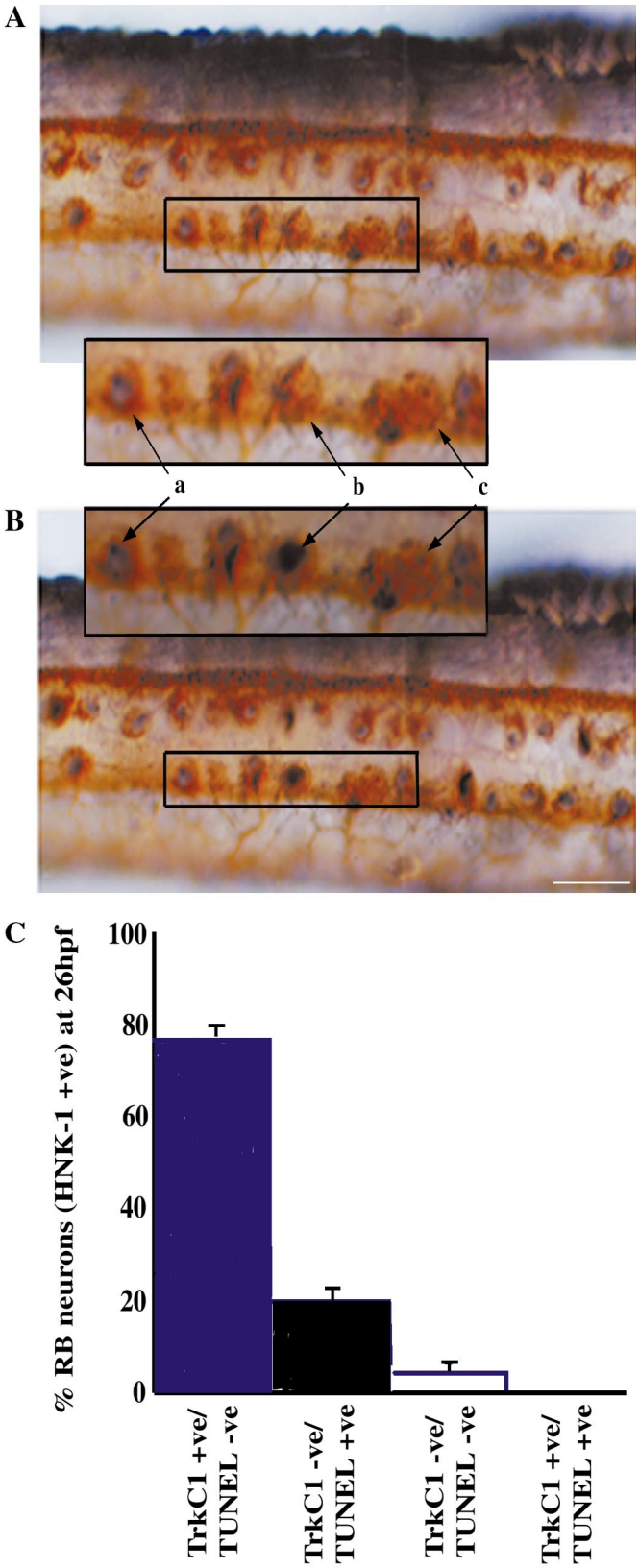


FIG. 2. Rohon Beard neurons die by caspase-dependent programmed cell death. (A–D) Dorsal views of spinal cords at 26 hpf (B–D) or a lateral view of a whole embryo at 28 hpf (A). (A) TUNEL labeling reveals a row of large apoptotic cells (arrow) within the dorsal spinal cord. (B) Double labeling with HNK-1 antibody (brown) and TUNEL (black) shows that a subpopulation of RB cells is dying. Two double-labeled cells are indicated with arrows. (C) zFAfmk (a control peptide)-treated embryo double labeled with HNK-1 (brown) and TUNEL (black). Double-labeled cells (arrows) are found in a frequency similar to that of wild-type embryos. (D) Caspase inhibitor zVADfmk-treated embryo double labeled with HNK-1 (brown) and TUNEL (black). There are no dying cells in this example. Scale bar, 25 μm. (E) Graph showing that the caspase inhibitor zVADfmk significantly reduces programmed cell death of RB cells in 26-hpf but not 48-hpf embryos compared to untreated and zFAfmk-treated controls. Values represent means ± SEM of five separate determinations. $^{**}P < 0.02$, according to the Student *t* test.

tions (Bernhardt *et al.*, 1990). As apoptotic RB neurons are first detected from 19 hpf, then it is clear that substantial RB cell death is occurring prior to differentiation of the DRG and certainly before the neurons in these ganglia innervate the skin. This suggests that competition between DRG and RB cells for target-dependent factors is unlikely to be the cause of early RB death.

Nonapoptotic RB Cells Express trkC1

RB cell death may be due to a loss of neurotrophins or neurotrophin receptors required for survival. In zebrafish, the neurotrophin receptor TrkC1 is expressed in a proportion of RB cells at 24 hpf (Martin *et al.*, 1998). To determine the proportion of RB cells that express *trkC1*, *in situ*



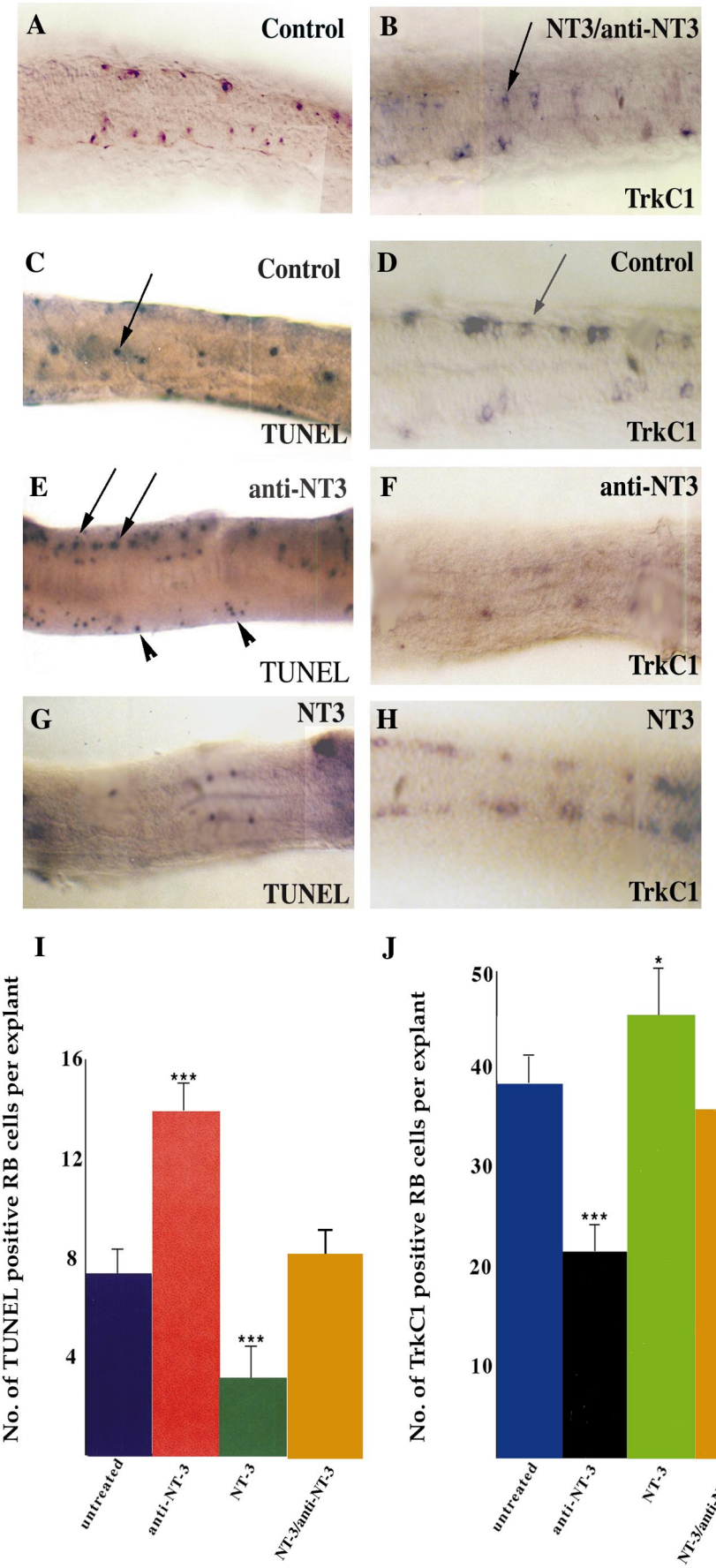
hybridization with a *trkC1* probe was combined with HNK-1 antibody to label RB cells in embryos between 15 and 26 hpf and *trkC1* cells were counted in singly labeled preparations at later stages. *trkC1* expression is first detected in RB cells at the 15-somite stage (16.5 hpf), and at early stages of development, the majority of RB cells are *trkC1* positive (Figs. 1F and 3A), whereas at later stages more of the neurons are apoptotic and fewer express *trkC1* (Fig. 1F).

To determine if there is a direct relationship between RB cell survival and *trkC1* expression, we compared expression of all three markers: HNK-1, *trkC1,* and TUNEL (Fig. 3B). At 26 hpf, we found that the *trkC1*/HNK-1-positive cells were not TUNEL positive (Fig. 3C). On average, 77% of the RB cell population were *trkC1* positive/TUNEL negative and 20% were TUNEL positive/*trkC1* negative ($n = 5$) (Fig. 3C). There was also a third population of RB neurons, about 3%, that were both *trkC1* negative and TUNEL negative (Fig. 3C). These results suggest that maintained *trkC1* expression is closely correlated with cell survival.

Anti-NT-3 Leads to Loss of trkC1 Expression and Increases RB Cell Death

To determine if the ligand for TrkC1, NT-3, is required for RB cell survival, we attempted to lower the levels of NT-3 available to the RB cells. To do this, a function-blocking antibody to NT-3 was applied to 16-somite stage trunk explants grown in culture for either 4 or 6 h. We used an anti-human NT-3 which has been shown to bind NT-3 in all species tested (Oncogene Research Products). A range of concentrations of blocking antibody between 0.5 and 200 ng/ml has been used previously by others in various model systems and in our experiments we present data using a concentration of 20 ng/ml. Explant cultures of trunk tissue were used to allow easy penetration of the antibody. RB neurons differentiate on schedule in these dissected trunk explants embedded in agar and grown in culture medium (Figs. 4A–4D). For the antibody-blocking experiments, *trkC1 in situ* hybridization was carried out on explants

FIG. 3. *trkC1* expression is restricted to Rohon Beard cells not undergoing apoptosis. (A and B) Equivalent dorsal views of spinal cords of the same 26-hpf embryo initially labeled with HNK-1 (brown) and a probe for *trkC1* (blue) and shown in (A). The embryo was then labeled by TUNEL to reveal dying cells (dark brown) and is shown again in (B). (A) *trkC1* is expressed in a subpopulation of RB cells. The inset indicates one *trkC1*-expressing HNK-1$^+$ neuron (a) and two HNK-1$^+$ neurons that do not express *trkC1* (b and c). (B) TUNEL labeling reveals that the *trkC1*-expressing RB cell (a) is not apoptotic. One of the HNK-1$^+$/TrkC1$^-$ cells (b) is apoptotic and one is not (c). None of the *trkC1*$^+$ neurons were double labeled by TUNEL. Scale bar, 25μm. (C) Graph showing the different percentages of TrkC1$^+$/TUNEL$^-$, TrkC1$^-$/TUNEL$^+$, TrkC1/TUNEL$^-$, and TrkC1$^+$/TUNEL$^+$ RB cells in 26-hpf embryos ($n = 5$).


A
Control
B
NT3/anti-NT3
TrkC1
C
Control
TUNEL
D
Control
TrkC1
E
anti-NT3
TUNEL
F
anti-NT3
TrkC1
G
NT3
TUNEL
H
NT3
TrkC1
I
No. of TUNEL positive RB cells per explant
16
12
8
4
***
***
untreated
anti-NT-3
NT-3
NT-3/anti-NT-3
J
No. of TrkC1 positive RB cells per explant
50
40
30
20
10
*
***
untreated
anti-NT-3
NT-3
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fixed after 4 h treatment and TUNEL was carried out on explants fixed after 6 h treatment. *trkC1*-positive cells were identified as RB cells as no other cells express *trkC1* in the zebrafish trunk and TUNEL-positive RB cells were distinguished from other dying cells by size and position.

Anti-NT-3 led to a reduction of *trkC1* expression (Figs. 4F and 4J) and induced increased programmed RB cell death in a dose-dependent manner compared to controls (Figs. 4E and 4I and data not shown). Other discrete populations of cells in addition to the RB neurons were also induced to die by anti-NT-3, including skin cells and other neurons of the spinal cord (data not shown).

Exogenous NT-3 Reduces RB Cell Death and Leads to an Increase in trkC1-Expressing RB Cells

To test if NT-3/TrkC1 signaling is sufficient to promote RB cell survival, trunk explants at the 16-somite stage were incubated with exogenous human NT-3 for 4 or 6 h (human NT-3 can activate zebrafish TrkC1; S. Martin, personal communication). A range of concentrations between 0.05 and 50 ng/ml was chosen from experiments in other model systems. Raising the level of NT-3 to 2 ng/ml in medium bathing the explants significantly decreased the number of RB cells undergoing cell death (Figs. 4G and 4I) and led to an increase in the number of RB cells expressing *trkC1* (Figs. 4H and 4J). However, application of NT-3 never prevented all the RB cells from dying, suggesting that other factors must play additional roles in promoting RB cell survival. When anti-NT-3 and NT-3 were applied together, the number of RB cells expressing *trkC1* and undergoing cell death was similar to that of untreated samples (Figs. 4B, 4I, and 4J).

DISCUSSION

In this paper we show that in zebrafish, the early sensory system RB cells die by apoptosis that is dependent upon NT-3 but is unlikely to be mediated through competition with the DRG that replace the RB cells. We show that this early RB cell death can be transiently reduced by the caspase inhibitor, zVADfmk. Furthermore, in cultured explants, death is also prevented by exogenous NT-3, while death is increased by blocking NT-3 signaling with function-blocking anti-NT-3 antibody.

DRG Innervation Does Not Control RB Cell Death

We have shown that in the majority of embryos, RB cells are removed between 20 hpf and 3.5 days of development. The removal of all RB cells by late larval stages also occurs in frogs, but in contrast, newts and lampreys retain functional RB cells throughout life.

Competition for survival factors between zebrafish RB cells and DRG neurons is unlikely to play a significant role in regulating RB cell death. There is extensive death of RB cells at stages well before any DRG neurons differentiate and during the period when DRG are thought to differentiate and innervate the periphery, there is no obviously increased RB cell death. Indeed, death of zebrafish RB neurons is slow and gradual, with no obvious waves of death, suggesting that a gradual depletion of survival factors could account for the continuous demise of the RB cells. DRG neurons could play a subtle role in late RB cell development, competing for the same factors as RB cells, but this cannot account for the majority of RB cell death. In contrast to fish, RB cells in *Xenopus* undergo two waves of cell death, one just before the DRG innervate the skin and a second just before metamorphic climax (Lamborghini, 1987). It has therefore been suggested that the first wave of RB cell death may be triggered through competition of RB cells and DRG for limited survival factors. However, even in *Xenopus*, RB neurons are not immediately superceded when the neurons of the DRG send fibers into the spinal cord and out to the periphery, and a proportion of RB cells are not affected (Lamborghini, 1987), suggesting that if the DRG do regulate the death of the RB neurons in frogs, they are probably not the only controlling factor.

One further difference in the death of RB cells in ze-

FIG. 4. Anti-NT-3 induces Rohon Beard cell death, whereas NT-3 reduces death and leads to an increase in *trkC1*-expressing neurons. (A–G) Dorsal views of 22-hpf (A, C, E, G) and 20-hpf (B, D, F, H) explant cultured spinal cords with anterior to the left. (A–D) Control preparations showing explants labeled to reveal HNK-1-expressing RB neurons (A), *trkC1*-expressing RB cells in an explant treated with anti-NT-3 (20 ng/ml) and NT-3 (2 ng/ml) (B, arrow indicates *trkC1*$^+$ cell), TUNEL-labeled apoptotic cells (C, arrow indicates large RB cell undergoing cell death), and *trkC1*-expressing RB cells in a control explant (D, arrow indicates *trkC1*$^+$ RB cell). (E) Explant treated with anti-NT-3 (20 ng/ml) for 6 h exhibiting an increased number of dying RB cells (arrows) compared to C, as determined with TUNEL. Some other cells in the skin and spinal cord are also dying (arrowheads). (F) Explant treated with anti-NT-3 (20 ng/ml) for 4 h exhibiting a decreased number of *trkC1*-expressing RB cells compared to D. (G) Explant treated with NT-3 (2 ng/ml) for 6 h exhibiting a decreased number of dying cells as determined by TUNEL compared to C. (H) Explant treated with NT-3 (2 ng/ml) for 4 h exhibiting an increased number of RB cells expressing *trkC1* compared to D. (I) Graph showing the number of TUNEL-positive RB cells per 26-somite stage (22 hpf) zebrafish explant in untreated, anti-NT-3-treated (20 ng/ml), NT-3-treated (2 ng/ml), and both anti-NT-3 (20 ng/ml) and NT-3 (2 ng/ml)-treated conditions. (J) Graph showing the number of *trkC1*-positive RB cells per 22-somite stage (20 hpf) zebrafish explant in untreated, anti-NT-3-treated (20 ng/ml), NT-3-treated (2 ng/ml), and both anti NT-3 (20 ng/ml) and NT-3 (2 ng/ml)-treated conditions. Values represent means ± SEM of five separate determinations $***P < 0.01$, $*P < 0.05$ according to the Student *t* test.

brafish compared to frogs is that in frogs RB cell death takes place in a craniocaudal progression, with the last surviving cells all found in the tail. Our studies show that in zebrafish, RB cell death occurs all along the trunk of the embryo from 19 h to 3.5 days. It is unknown why such differences might occur although they are also reflected in the timing of differentiation of spinal cord neurons. Reflecting the random distribution of dying cells along the AP axis, there are also no obvious craniocaudal waves of neuronal differentiation in the trunk spinal cord of fish embryos (Bernhardt *et al.,* 1990).

RB Neurons Undergo Caspase-Dependent Programmed Cell Death

The caspase inhibitor zVADfmk reduces cell death of RB cells up until 36 h of development but after this time, the drug offers no protection and eventually all the zebrafish RB cells die. Caspase inhibitors have previously been shown to prevent cell death in motor neurons *in vivo* and *in vitro* (Milligan, 1995) and to prevent apoptosis of NGF-deprived DRG neurons (Mukasa, 1997). However, zVADfmk produces only a significant delay of death rather than providing a permanent and complete rescue of RB neurons. This temporary effect has been shown to occur in other neurons (Kermer *et al.,* 1999). The failure of zVADfmk to permanently rescue the RB neurons could be due to the activity of the caspase inhibitor not being irreversible or, alternatively, there is evidence that in some cases, rescued cells will eventually die via a necrotic route (Lemaire *et al.,* 1998). Repetitive additions of zVADfmk did not continue to prevent cell death although we cannot discard the possibility that permeability problems at late stages in zebrafish development prevented the drug from reaching the neurons. Unfortunately, it proved not possible to use cultured explants for sufficient periods of time to resolve this issue.

trkC1 Expression Correlates with RB Survival

We found that the neurotrophin receptor *trkC1* is expressed only in RB cells that are not labeled by TUNEL, i.e., not undergoing apoptosis. This raises the possibility that loss of *trkC1* receptor signaling may contribute to the induction of RB neuron apoptosis. Without being able to trace gene expression over time in individual cells, we cannot definitely resolve the temporal sequence of events leading to death. However, several observations lead us to believe that loss of *trkC1* expression may be a trigger of death rather than a consequence of death.

The majority of differentiated (HNK-1$^+$) RB cells that did not express *trkC1* were apoptotic but we observed a consistent small proportion of such neurons that were not TUNEL labeled. It is tempting to speculate that these are neurons in which *trkC1* has been downregulated but that apoptotic changes have yet to be initiated. Second, TUNEL detects cells early in the process of programmed cell death and it seems doubtful that all *trkC1* mRNA in the cell would have undergone turnover between the time that apoptosis is initiated and the time that TUNEL labels the nucleus. Indeed, it has been shown that certain mRNAs are present in all RB cells, which must include those that are undergoing cell death (Munchberg *et al.,* 1999), suggesting that a complete loss of mRNAs does not precede TUNEL labeling during the process of apoptosis. Therefore it seems most probable that if signaling via TrkC1 is prevented, RB cell death occurs. This is also supported by the fact that when exogenous NT-3 (likely to be the main ligand for TrkC1) is sequestered by anti-NT-3, the number of *trkC1*-positive RB cells decreases and an increase in death occurs.

trkC1 downregulation in certain RB cells could be part of a defined intrinsic developmental program or could be due to alterations in extrinsic factors (such as levels of NT-3 in the embryo). In the former case, the acquisition and loss of responsiveness to different neurotrophins may be associated with changes in the expression of the corresponding receptors. In the latter, death and the downregulation of the receptor may be directly consequent upon the level of the neurotrophin ligand.

NT-3 Is Necessary for RB Survival in Culture

In explant cultures, reducing endogenous NT-3 with function-blocking antibodies induces RB cell death while exogenous application of NT-3 reduces RB death. This suggests that *in vivo,* NT-3 may be available in limiting amounts and may be a primary extrinsic regulator of RB cell survival and death.

To determine the physiological, rather than the pharmacological, response of RB cells to NT-3, it will be necessary to determine where and when NT-3 is present during development. NT-3 has yet to be cloned in zebrafish so it is not yet possible to determine its spatial and temporal patterns of expression. Furthermore, antibodies to NT-3 from other species have not been successful for immunochemistry in zebrafish. In other species, mRNA for NT-3 is initially most highly expressed in the mesenchyme through which sensory axons extend to the periphery and is later confined to the mesenchyme underlying the skin (Buchman and Davies, 1993; Wilkinson *et al.,* 1996). These are regions that would be likely sources of NT-3 for RB cell axons.

While it seems very likely that endogenous NT-3 will signal through TrkC1 in zebrafish as in other species, it is possible that NT-3 could also act as a survival factor through other receptors. For instance, application of anti-NT-3 induced death in non-RB cells which do not express TrkC1, suggesting that NT-3 can indeed act through other receptors in zebrafish. This is in accordance with the phenotype of NT-3-null mice, which have a more severe phenotype compared to TrkC-null mice, implying that TrkC is not the only receptor for NT-3 (Liebl *et al.,* 1997). It may also be possible that other neurotrophins mediate the survival of RB neurons, so while we have shown that NT-3 is necessary for RB survival, it may not be sufficient.

In summary, we have shown that a decrease of *trkC1*

expression correlates with induction of RB cell death and that by sequestering NT-3, RB cell death can be induced. Our current hypothesis is that lowered levels of NT-3 in the extracellular environment of the RB cells may lead to lowered levels of expression of its cognate receptor, TrkC1, thus amplifying the decreased level of neurotrophin signaling. Alternatively *trkC1* may be intrinsically downregulated in RB neurons. We suggest that lowered neurotrophin signaling subsequently induces caspase-dependent apoptosis within the RB neurons.

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